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Glycoproteomic Analyses of Ovarian Cancer Cell Lines and Sera from Ovarian Cancer Patients Show Distinct Glycosylation Changes in Individual Proteins

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Ovarian cancer is difficult to diagnose in women because symptoms of the disease are often not noticed until the disease has progressed to an advanced untreatable stage. Although a serum test, CA125, is currently available to assist with monitoring treatment of ovarian cancer, this test lacks the necessary specificity and sensitivity for early detection. Therefore, better biomarkers of ovarian cancer are needed. A glycoprotein analysis approach was undertaken using high resolution Fourier transform ion cyclotron resonance mass spectrometry to analyze glycosylated proteins present in the conditioned media of ovarian cancer cell lines and in sera obtained from ovarian cancer patients and normal controls. In this study, glycosylated proteins were separated by gel electrophoresis, and individual glycoproteins were selected for glycosylation analysis and protein identification. The attached glycans from each protein were released and profiled by mass spectrometry. Glycosylation of a mucin protein and a large glycosylated protein isolated from the ES2 ovarian cancer cell line was determined to consist of mostly *O*-linked glycans. Four prominent glycoproteins of approximate 517, 370, 250, 163 kDa from serum samples were identified as two forms of apolipoprotein B-100, fibronectin, and immunoglobulin A1, respectively. Mass spectrometric analysis of glycans isolated from apolipoprotein B-100 (517 kD) showed the presence of small, specific *O*-linked oligosaccharides. In contrast, analysis of fibronectin (250 kD) and immunoglobulin A1 (163 kD) produced *N*-linked glycan fragments in forms that were sufficiently different from the glycans obtained from the corresponding protein band present in the normal serum samples. This study shows that not only a single protein but several are aberrantly glycosylated, and those abnormal glycosylation changes can be detected and may ultimately serve as glycan biomarkers for ovarian cancer.

Keywords: ovarian cancer • human serum • cancer marker • glycoprotein • glycan • SDS-PAGE electrophoresis • MALDI mass spectrometry

Introduction

Ovarian cancer has a high morbidity rate, largely because it is difficult to diagnose in the early stages of the disease. Although a serum test for CA125 is available for monitoring the disease, this test lacks the specificity and sensitivity for early detection.[1] Better methods involving mass spectrometry to perform molecular analysis of the serum are being developed and may provide more robust biomarkers of ovarian cancer.[2] However, most studies are focused on analysis of total serum proteins. Glycosylation of tumor proteins is known to become significantly altered in cancers.[3] Extensive investigations on glycoproteins in tumor cells have demonstrated that the malignant transformation of tumor cells is associated with various and complex alterations in the cell glycosylation process.[4] Altered glycosylation can also be observed as significant changes on individual proteins as shown recently on prostate-specific antigen (PSA)[5] and human pancreatic ribonuclease 1 (RNase 1).[6] Specifically, normal PSA was found to contain more sialylated *N*-glycans compared to those found in PSA produced by a human prostate tumor cell line (LNCaP), where the LNCaP PSA contained only neutral glycans and a higher fucose content;[5] the fucose content of PSA from the prostate cancer patient serum was significantly lower and there was a decrease in α2,3- linked sialic acid in comparison with normal plasma PSA.[7] RNase 1 was found to contain an increased level (by as much as 40%) of core fucosylation in the

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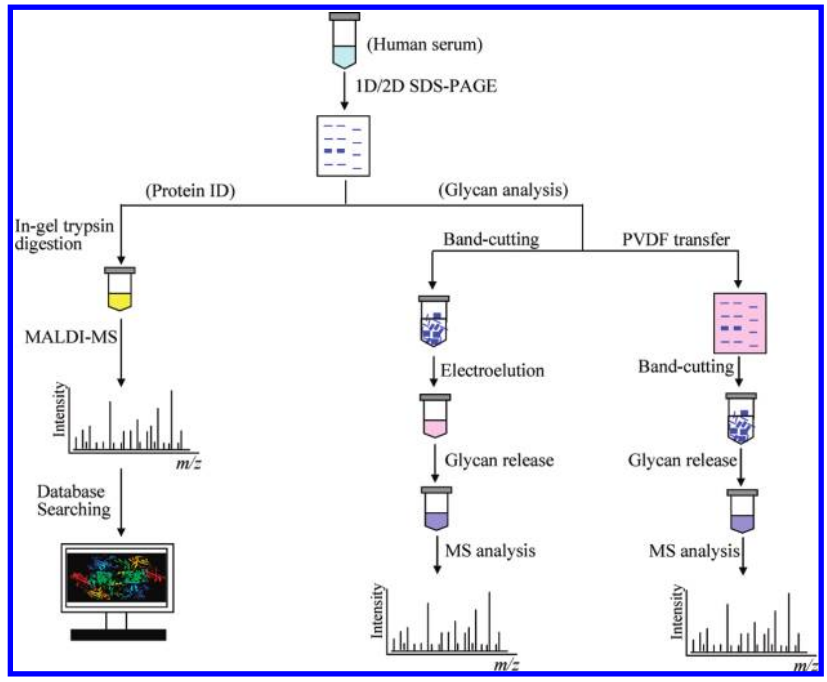
Scheme 1. Schematic Flow Chart of Procedures for Glycoprotein Identification and Glycan Analysis for Ovarian Cancer Cell Lines and Human Serum Samples



main sialylated biantennary glycans in serum of patients with pancreatic cancer compared to the normal serum although both normal and tumor serum RNase 1 contained the same glycan structures.[8] Previously, Lewis glycan antigen structures sialyl-Lewis^x (SLe^x) and sialyl-Lewis^a (SLe^a) were reported to be increased in carcinoma cells.[9] The increase in the glycan structures may provide a selective advantage for tumor cells and help them to evade immune response during their progression to more invasive and metastatic phenotype.[10]

Glycan biosynthesis and diversity are produced by the cellular "glycosylation machinery" that is composed of complex additions of monosaccharides through the actions of dolichol accessory molecules present in the endoplasmic reticulum (ER) and glycosyltransferases present in ER and the Golgi. The machinery is sensitive to malignant transformation and is responsible for glycosylation changes associated with cancers.[11,12] Therefore, glycoproteins produced by the malignant cell may all potentially be misglycosylated.

In cancers, the *O*-glycans, especially those of mucin-type glycoproteins, play a significant role in recognition, attachment and invasion during cancer growth and metastasis.[13] *O*-glycosylation of proteins can be changed structurally and quantitatively in tumor cells.[14] The biosynthesis of glycans involves enzyme families that compete to yield different linkages because the substrate specificities of *O*-glycosyltransferases are relatively broad. If a glycosyltransferase is down regulated, then it is outcompeted by others resulting in the aberrant glycosylation of the nascent chain. The subsequent glycosyltransferase in the machinery therefore adds to the aberrant glycan resulting eventually in an altered *O*-glycan structure.[14]

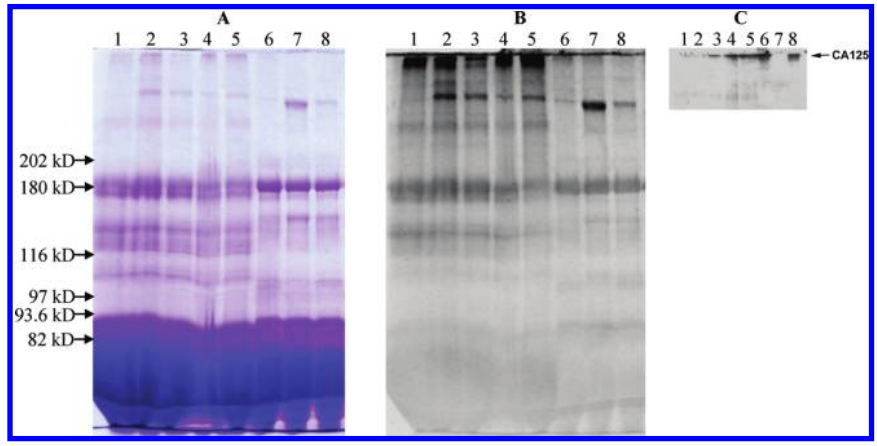


Figure 1. SDS-PAGE gels (6%) for ovarian cancer cell lines and human serum samples were stained with (A) Coomassie blue for total proteins and (B) ProQ Emerald 300 for glycoproteins. (C) Western blot analysis of a duplicate gel probed with CA125 antibody for CA125. Lane 1: medium DMEM; lanes 2−5: cell line supernatant, lane 2: SKOV3; lane 3: ES2; lane 4: OVCAR; lane 5:CaOV3. Lanes 6−8: human serum, lane 6: ovarian cancer patient #11 (CA125 was 382); lane 7: ovarian cancer patient #12 (CA125: 755); lane 8: normal serum #22 (CA125: 15).

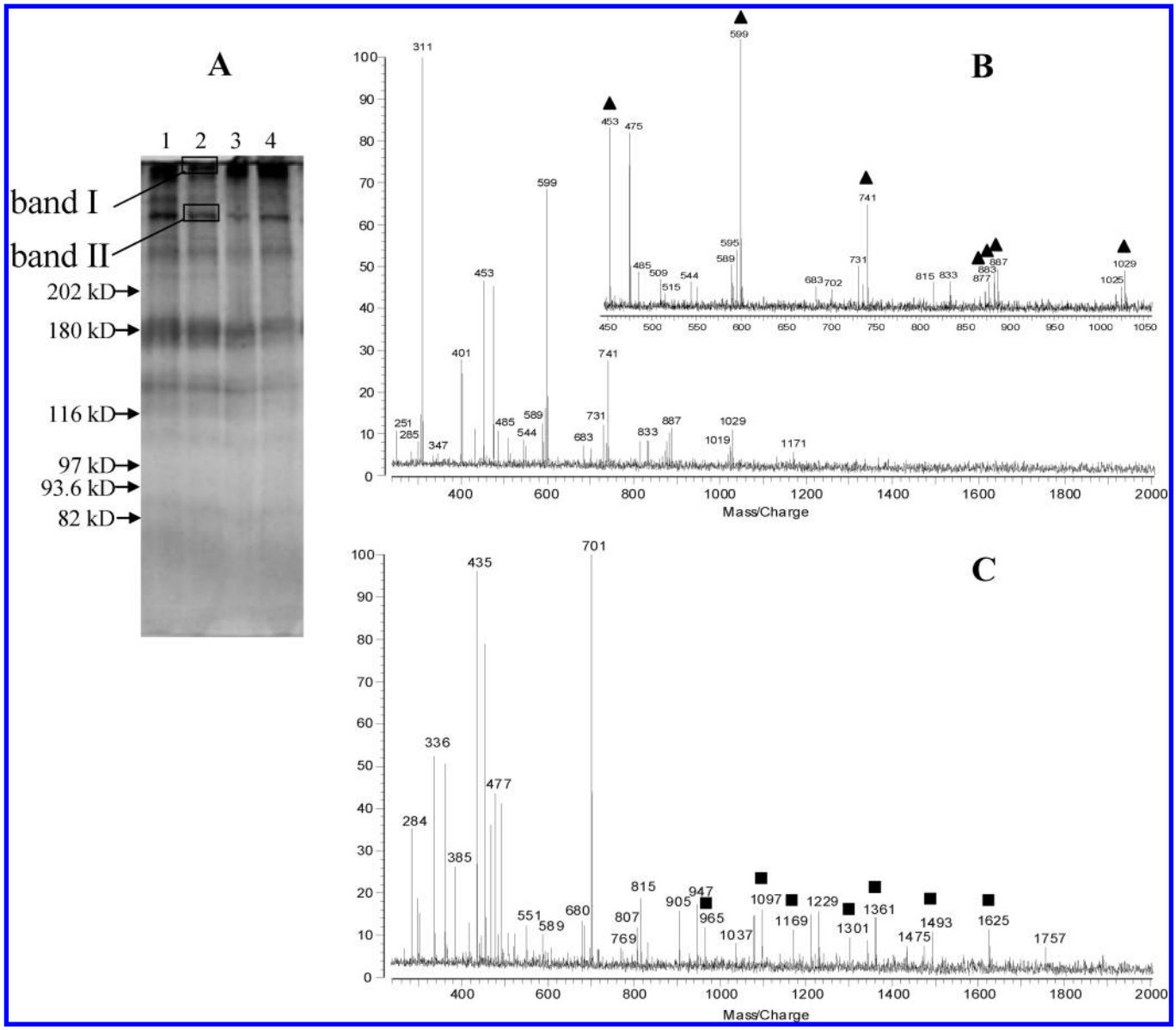


Figure 2. (A) MALDI mass spectra of glycans released from glycoprotein bands in the supernatant of ovarian cancer cell line, ES2. (B) Mass spectrum for band **I** and (C) for band **II**. Lanes 1−4: cell line supernatant, lane 1: SKOV3; lane 2: ES2; lane 3: OVCAR; lane 4: CaOV3. Symbols, ▲ and ■, represent glycan peaks in the spectra (B) and (C), respectively.

The most frequently reported tumor-associated changes in glycosylation include an increase in the branching of *N*-glycans, an increase in sialylation and polysialic acid synthesis, the appearance of Lewis-type antigens in glycoproteins and glycolipids, and a shortening of *O*-glycan chains.[15,16] Recently Ueda et al. reported, using a comparative glycoproteome profiling approach of human serum, that a total of 34 serum glycoproteins were identified as potential biomarker candidates for lung cancer. They were obtained by monitoring the changes of α1,6-fucose levels of the residue attached to the asparagines-linked core GlcNAc residues with a *Lens culinaris* (LCA) lectin column.[17] Among the 34 identified glycoproteins, there were 20 with more than 2-fold increase of α1,6-fucose level and 14 with less than 2-fold decrease of the residue compared to the healthy controls.[17]

Although glycans have received less attention as potential cancer markers than peptides and proteins, the use of glycans as biomarkers has certain distinct advantages. By focusing on glycosylated proteins, the number of glycans and proteins in the pool of potential cancer markers would be significantly smaller. Using recently described methods employing high resolution mass spectrometry analysis of human serum for ovarian[18] and breast cancers,[19] glycans were readily identified and quantified. Glycosylation occurs mainly in the ER and the golgi. Aberrant glycosylation could affect multiple proteins in the same cell.[20]

In this study, a combined glycan and protein identification approach was used to monitor and detect glycosylation changes of specific glycoproteins in ovarian cancer cell lines and in cancer and normal sera. Due to its high resolution, high sensitivity and high mass accuracy, Fourier transform ion cyclotron resonance (FT-ICR) with matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) represents a powerful analytical tool in structure elucidations of especially glycans.[21] The results demonstrate that although some protein regulations may be unchanged or slightly changed between

Table 1. List of Positive Oligosaccharides Found in an Ovarian Cancer Cell Line, ES2 (Figure *2*)

band	observed mass (*m*/*z*)	oligosaccharide composition[a]	quasimolecularion
band I	453.0726	1Hex:1Fuc:1Sulfate	$(M - H + 2Na)^+$
	599.2885	1Hex:2Fuc:1Sulfate	$(M - H + 2Na)^+$
	741.2391	2Fuc:2HexNAc	$(M + Na)^+$
	877.2684	2Hex:1HexNAc:1NeuGc	$(M + Na)^+$
	883.2690	1Hex:1Fuc:1HexNAc:1NeuGc or 2Hex:1HexNAc:1NeuAc	$(M - H + 2Na)^+$
			$(M - H + 2Na)^+$
	887.4612	3Fuc:2HexNAc	$(M + Na)^+$
	1029.4151	2Hex:1Fuc:1HexNAc:1NeuAc or 1Hex:2Fuc:1HexNAc:1NeuGc	$(M - H + 2Na)^+$
			$(M - H + 2Na)^+$
band II	965.2757	3HexNAc:1NeuAc	$(M - H + 2Na)^+$
	1097.3210	4Hex:2HexNAc or 1Hex:4HexNAc:1Sulfate	$(M + Na)^+$
			$(M + Na)^+$
	1169.3334	3Hex:1Fuc:1HexNAc:1NeuAc or 2Hex:2Fuc:1HexNAc:1NeuGc	$(M + Na)^+$
			$(M + Na)^+$
	1301.3613	4Hex:2HexNAc:2Sulfate	$(M - 2H + 3Na)^+$
	1361.3883	5Hex:2HexNAc:1Sulfate	$(M - H + 2Na)^+$
	1493.4126	5Hex:1Fuc:1HexNAc:1NeuAc or 4Hex:2Fuc:1HexNAc:1NeuGc	$(M + Na)^+$
			$(M + Na)^+$
	1625.4333	6Hex:2HexNAc:2Sulfate	$(M - 2H + 3Na)^+$

[a] All oligosaccharides listed here are in alditols—the residue at reducing end of each glycan is alditol.

Table 2. Summary of Glycoprotein Identifications with Peptide Mass Fingerprinting (PMF) of Gel Bands (Figure *3*A) from Human Serum Samples

gel band in Figure *3*A	NCBInr access no.	protein name	Mascot score[a]	molecular weight (Da)	peptides matched	sequence coverage
band a	gil109658664	Fibronectin 1	80	243063	34[b]	13
	gil47132549	Fibronectin 1 isoform 6 preproprotein	80	243078	34	13
	gil30722344	Hypothetical protein (Fibronectin precursor, differentially spliced)	78	252738	35	12
	gil53791223	Fibronectin 1	76	252848	34	12
	gil47132553	Fibronectin 1 isoform 5 preproprotein	75	256255	34	12
	gil47132555	Fibronectin 1 isoform 4 preproprotein	75	259943	35	12
	gil16933542	Fibronectin 1 isoform 3 preproprotein	74	262656	35	12
	gil62089266	Fibronectin 1 variant	73	262270	34	12
	gil47132551	Fibronectin 1 isoform 2 preproprotein	72	269633	35	12
band b	gil178812	Apolipoprotein B-100 precursor	66	516445	33	7
band c	gil178818	Apolipoprotein B-100	64	370484	18	7

[a] Protein scores greater than 64 are significant ($p < 0.05$). [b] As an example, the 34 matched peptides are summarized in Chart 1.

normal and disease, glycosylation changes are more evident and may be a better indicator for the onset of the disease.[22] This study provides further evidence that glycomic analysis can play an important role in the identification of cancer markers.

Experimental Section

The experimental design is outlined in Scheme 1. Briefly, human serum proteins were separated with 6% SDS-PAGE. The separated proteins were then subjected to both protein identification using peptide mass fingerprinting approach and glycan analysis.

Ovarian Cancer Cell Growth and Supernatant Acquisition. ES-2 and SK-OV-3 cells were grown in McCoy's media. Caov-3 and OVCAR-3 cancer cells were cultured in RPMI1640 cell media supplemented with 10% fetal bovine serum, 100 units/mL penicillin/streptomycin and 1% glutamine. Conditioned media (CM) was removed from the cells during log (nonconfluent) or death (confluent) cell growth, and frozen at −70 °C. The CM was thawed, sterile filtered (0.2 μm filter), and concentrated using Vivaspin 20 concentrator (VivaScience, Edgewood, NY). Alternatively, the filtered CM was dialyzed (MWCO: 10 KDa) extensively against distilled water and then lyophilized.

Human Serum Samples. Serum samples were obtained from female individuals in a normal control group (without a known history of ovarian, primary peritoneal cancers, any other malignancies, significant medical or surgical problems) and from patients diagnosed with ovarian cancer (UC Davis Medical Center). The controls were selected to be within the same age range as the patient samples. All samples were pre-existing samples that were deidentified by the Pathology Department before analysis. Both the controls and cancer patient samples were tested for CA125 measurements using the AXSYM test (Abbott, Abbott Park, IL) by the Specialty Chemistry Unit, UC Davis Medical Center Clinical Laboratories. For the glycoprotein identification and glycan analyses of human serum, pooled sera

Chart 1. Summary of the 34 Matched Peptides for *fibronectin 1* at First Entry of Band a in Table 2

Observed	Mr(expt)	Mr(calc)	ppm	Start		End	Miss	Peptide
146.1054	146.1054	146.1055	-0.79	1900	-	1900	0	K.K.T
174.1115	174.1115	174.1117	-0.94	1729	-	1729	0	R.R.A
231.1329	231.1329	231.1331	-1.01	1352	-	1353	0	R.GR.Q
245.1486	245.1486	245.1488	-0.71	1838	-	1839	0	R.AR.I
273.1802	273.1802	273.1801	0.42	1973	-	1974	0	K.VR.E
287.1959	287.1959	287.1957	0.61	1390	-	1391	0	R.IR.H
502.2501	502.2501	502.2499	0.32	1888	-	1891	0	K.NNQK.S
563.3442	563.3442	563.3431	1.94	641	-	644	0	K.YILR.W
655.3433	655.3433	655.3442	-1.36	1017	-	1021	0	R.WTPPR.A
746.3741	746.3741	746.3711	3.97	150	-	155	0	K.IGDTWR.R
758.4756	758.4756	758.4650	13.9	1029	-	1035	0	R.LTVGLTR.R
770.4245	770.4245	770.4286	-5.37	1892	-	1898	0	K.SEPLIGR.K
780.4138	780.4138	780.4130	1.03	1383	-	1389	0	R.ATITGYR.I
807.4261	807.4261	807.4239	2.74	1022	-	1028	0	R.AQITGYR.L
860.4106	860.4106	860.4028	9.03	480	-	486	0	R.IGDQWDK.Q
871.4674	871.4674	871.4664	1.09	977	-	984	0	K.VFAVSHGR.E
1029.5335	1029.5335	1029.5243	8.91	1847	-	1855	0	K.YEKPGSPPR.E
1109.5286	1109.5286	1109.5353	-6.05	1198	-	1207	0	R.STTPDITGYR.I
1168.4535	1168.4535	1168.4430	8.99	585	-	592	0	R.YQCYCYGR.G
1220.5213	1220.5213	1220.5132	6.64	370	-	379	0	R.TFYSCTTEGR.Q
1274.6284	1274.6284	1274.6044	18.8	2091	-	2100	0	K.TYHVGEQWQK.E
1290.7383	1290.7383	1290.7183	15.5	1955	-	1966	0	R.GATYNIIVEALK.D
1322.7016	1322.7016	1322.7055	-2.94	1117	-	1129	0	K.LGVRPSQGGEAPR.E
1348.6897	1348.6897	1348.6776	9.01	1562	-	1573	0	K.WLPSSSPVTGYR.V
1354.7025	1354.7025	1354.6881	10.6	1791	-	1801	0	K.IYLYTLNDNAR.S
1400.6550	1400.6550	1400.6585	-2.52	58	-	67	0	K.HYQINQQWER.T
1430.7435	1430.7435	1430.7419	1.14	831	-	842	0	R.WSRPQAPITGYR.I
1482.6883	1482.6883	1482.6925	-2.86	504	-	515	0	R.GEWTCIAYSQLR.D
1592.8039	1592.8039	1592.8046	-0.45	1732	-	1745	0	R.VTDATETTITISWR.T
1628.8761	1628.8761	1628.8635	7.76	939	-	953	0	R.VDVIPVNLPGEHGQR.L
1650.8001	1650.8001	1650.7759	14.6	2021	-	2034	0	K.LLCQCLGFGSGHFR.C
1725.8001	1725.8001	1725.8145	-8.33	398	-	411	0	K.YSFCTDHTVLVQTR.G
1731.9358	1731.9358	1731.9407	-2.81	1055	-	1070	0	R.NLQPASEYTVSLVAIK.G
1925.0711	1925.0711	1925.0411	15.6	1285	-	1301	0	R.VTWAPPPSIDLTNFLVR.Y

(8 normal controls and 8 ovarian cancer patients) were used. The CA125 levels were 37, 23, 15, 46, 24, 15, 19, 12 for 8 normal sera; 17044, 241, 382, 755, 246, 361, 672, 1248 for 8 patients. The serum samples were aliquoted into 200 μL each and dialyzed against deionized water with Slide-A-Lyzer (MWCO 7K-10K, Pierce, Rockford, IL) at 4 °C overnight and lyophilized. The dried serum samples were stored at −80 °C.

SDS-PAGE Electrophoresis of Cancer Cell Lines and Human Serum Samples. Gels containing 6% acrylamide (separating) and 4% acrylamide (stacking) were prepared from ProtoGel of 30% (w/v) acrylamide/methylene bisacrylamide solution (37.5:1, National Diagnostics, Atlanta, GA), Tris/HCl (pH 8.8, final 0.375 M) and 10% sodium dodecyl sulfate (SDS, final 0.1%) based on published protocols.[23] The mixture was degassed for 15 min before tetramethylethylenediamine (TEMED) and ammonium persulfate added to the final concentrations of 0.05 and 0.0375%, respectively. Protein concentrations of cell line supernatants or human serum were determined (BioRad Protein Assay, BioRad, Hercules, CA) and equivalent amounts of protein were loaded per lane. Glycosylated protein standards (CandyCane Glycoprotein Molecular Weight Standards, Molecular Probes, Inc., Eugene, Oregon) and protein standards (Prestained protein standards, Invitrogen, Carlsbad, CA) were included.

For visualization of glycoproteins, gels were stained with Pro-Q Emerald 300 Glycoprotein Gel Stain Kit (Molecular Probes, Inc., Eugene, OR) following their procedure. The Pro-Q 300 stained gels were scanned using the Gel DOC 1000 system (Bio-Rad, Hercules, CA). To visualize total proteins, gels were stained with RAPID Stain Reagent (CALBIOCHEM, San Diego, CA) and the gels were scanned using a flatbed color scanner.

In-Gel Tryptic Digestion of Glycoproteins. The glycoprotein band of interest was excised from the gel and rinsed three times with Milli-Q water. The band was then cut into approximately 1 mm^2 pieces and dried. The gel slices were reduced with 250 μL of 10 mM dithiothreitol (DTT, Sigma-Aldrich) at 55 °C for

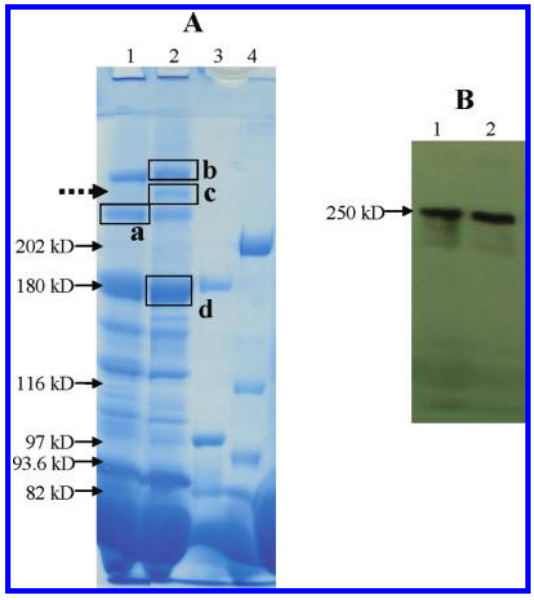


Figure 3. (A) Four glycoprotein bands (a, b, c, d, enclosed in rectangles) in 6% SDS-PAGE gel were subjected to in-gel tryptic digestion. (B) Western blot for the 250 kD band (corresponding to band a) for both pooled normal control and ovarian cancer sera with fibronectin monoclonal antibody mAb-1. The dotted arrow shows that band c is missing in the cancer sample. Lane 1: pooled patient sera; lane 2: pooled normal control sera; lane 3: CandyCane glycoprotein molecular weight standards; lane 4: prestained SDS-PAGE standards.

60 min and subsequently cooled down to room temperature. The liquid was extracted and 300 μL of 55 mM iodoacetamide (Sigma-Aldrich) was added for alkylation of reduced proteins in the dark at room temperature for 45 min. The gel pieces were washed with 100 mM NH_4HCO_3 by gently turning over several times followed by removal of liquid. Acetonitrile (AcN) was added in and then discarded as soon as gels got dehydrated and shrunk. The gel slices were completely dried under speed vacuum and added to a trypsin solution (5 μg in 50 mM NH_4HCO_3). The mixture was incubated at 37 °C overnight for digestion.

After the reaction was cooled down to room temperature, the supernatant was removed and saved. The gel was subsequently extracted with enough volume of 0.1% trifluoroacetic acid (TFA) and 5% formic acid in 50% AcN by gently mixing and incubation at 37 °C for 30 min, respectively. Each wash was combined with the saved supernatant, and the resulting solution was concentrated to about 50 μL. Tryptic peptides eluted from gels were desalted and concentrated on C_{18} Zip-tip (Millipore Corp, Billerica, MA).

Immunoblot Analysis for Glycoproteins. The protein bands were transferred onto Immuno-Blot polyvinylidene fluoride (PVDF) membrane (Bio-Rad) using semidry electrophoretic transfer cell (Bio-Rad). For the maximum transfer efficiency the gel/membrane was sandwiched by six pieces of thick filter paper saturated with transfer buffer and the transfer performed at 25 V for 2 h. For the Western protein analysis, the blotted PVDF membrane was incubated in 5% gelatin (in TBST, tris-buffered saline with 0.1% Tween 20) 1 h at room temperature with gentle shaking. After three washes, each 10 min with TBST, the membrane was first incubated with primary antibodies, Fibronectin Ab-1 (from Laboratory Vision Corporation, Fremont, CA) for fibronectin, or clone OC 125 antibody (from Zymed, South San Francisco, CA) against CA125, respectively, at 4 °C overnight with shaking followed by the reaction with enzyme-antibody conjugate, antimouse IgG conjugated with horseradish peroxidase (HRP), for one hour at room temperature. After the wash with TBST, the membrane was incubated in Western Lightning Chemiluminescence HRP substrate (Perkin-Elmer, Boston, MA) for one minute immediately before exposure to Kodak film for imaging.

Electroelution of Glycoproteins from Gel Bands. The glycoprotein bands of interest were excised after electrophoresis and enclosed in dialysis membrane (MWCO 3,500, diameter 11.5 mm, Spectrum Laboratories, Inc., Rancho Dominguez, CA) filled with electrode buffer and subjected to electrophoresis overnight at 30 mA. The resulting solution was dialyzed against deionized water at room temperature for 24 h. The supernatant was carefully transferred into a 15 mL tube while avoiding traces of gel. Solvents were removed by speed-vacuum evaporation.

Reductive Release of Glycans from Glycoproteins. For glycoproteins on PVDF membrane, the band of interest was cut into tiny pieces. About 200 μL (or 50 μL for dried glycoproteins) of solution containing 1.0 M $NaBH_4$ and 0.1 M NaOH was added in each tube containing PVDF pieces or dried glycoprotein residue after electroelution. The tubes were incubated at 42 °C for 24 h followed by careful neutralization with 1.0 M HCl acid.

The resulting *O*-linked oligosaccharide mixture was desalted and purified by the solid phase extraction (SPE) using nonporous graphitized carbon cartridge (Alltech, Lancaster, PA). The cartridge was first washed with deionized water then with 80% (v/v) acetonitrile−0.1% (v/v) trifluoroacetic acid followed by water. The oligosaccharide sample was loaded on the SPE cartridge and washed with water to remove salts. Oligosaccharides were eluted sequentially by 10, 20, and 40% (the latter with 0.05% trifluoroacetic acid (v/v)) acetonitrile in water. Each fraction was collected and dried (SpeedVac) prior to MALDI analysis.

Glycan Analysis by MALDI-FTICR Mass Spectrometry. A commercial MALDI-FT mass spectrometer (Varian Inc., Lake Forest, CA) with an external ion source was used to perform the mass spectrometric analysis. The instrument is equipped with a 7.0-T shielded, superconducting magnet and a Nd:YAG laser with working wavelength at 355 nm. MALDI sample was prepared by loading 1−6 μL of the analyte, 1 μL of 0.4 M matrix, 2,5-DHB, in 50:50 H_2O/acetonitrile on a stainless steel target plate as previously reported.[21] For the positive mode mass spectrometry, 1 μL of 0.1 M NaCl in 50:50 H_2O/acetonitrile was used as a cation dopant. The plate was allowed to air-dry before insertion into the ion source.

Peptide Mass Fingerprinting (PMF) for Protein Identifications. After in-gel tryptic digestion of glycoproteins and subsequent mass spectrometric analysis by MALDI FT-ICR MS, protein identifications were performed with Mascot (www.matrixscience.com) database search. The search parameters were mass error at <20 ppm, the number of missed cleavages with none and the score greater than 64 with 95% confidence.

Results and Discussion

We are developing new methods to analyze glycosylation of individual proteins obtained from ovarian cancer samples. The present work complements our previous studies where total glycosylation was monitored without protein identification.[18,24] We examined first conditioned media (cell supernatants) of

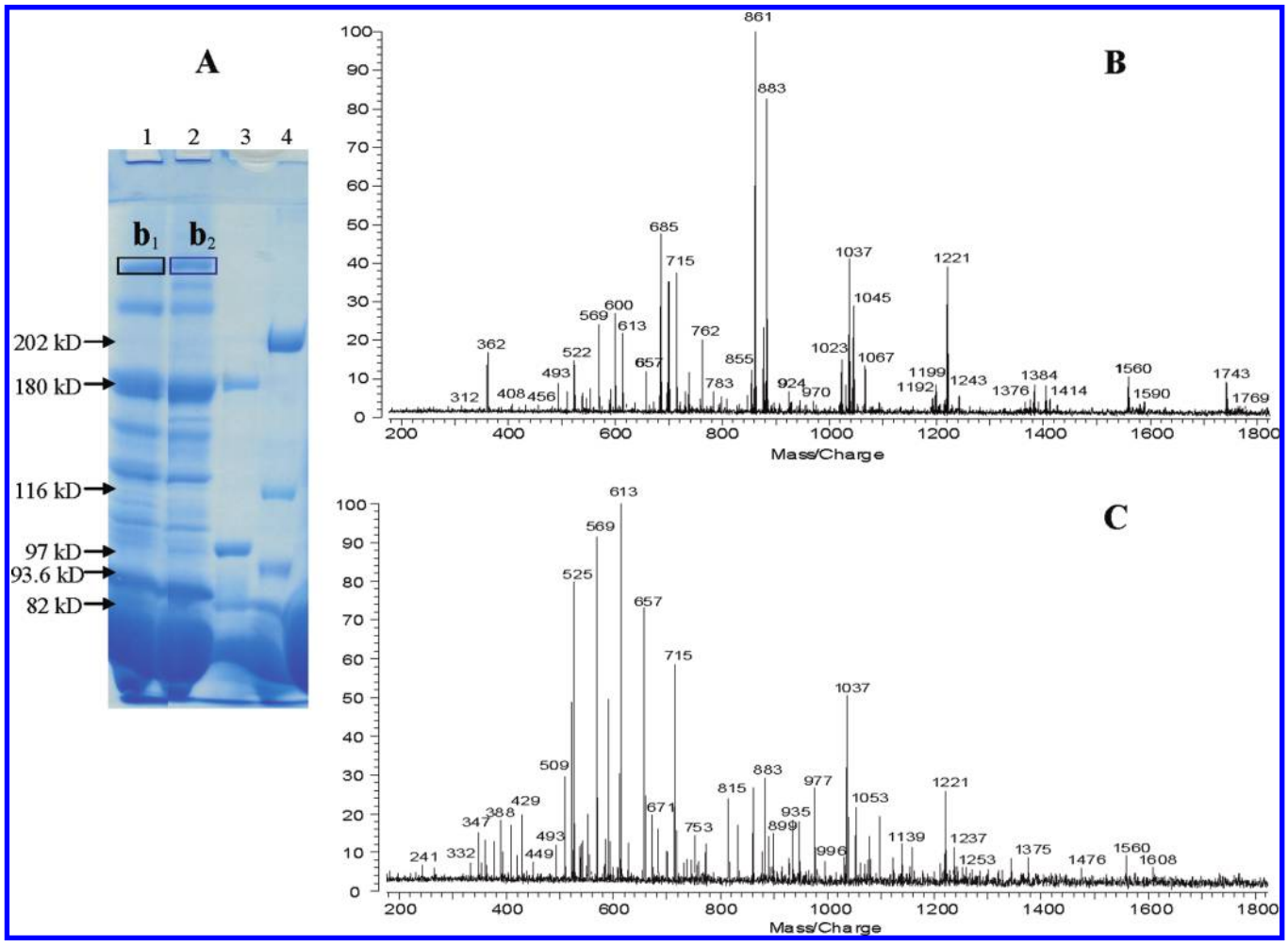


Figure 4. MALDI mass spectra of glycans released from glycoproteins of 517 kD (apolipoprotein B-100 gel bands, b_1 and b_2, in A) for normal (B) and patient sera (C). Glycan assignments and compositions are listed in Table 3. Glycoproteins were electroeluted out of gel bands. Lane 1: pooled patient sera; lane 2: pooled normal sera; lane 3: CandyCane glycoprotein molecular weight standards; lane 4: prestained SDS-PAGE standards.

ovarian cancer cell lines to observe changes in glycosylation of glycoproteins shed by the tumor cells.[18]

There are two major types of glycosylation in proteins, *O*- and *N*-linked. *O*-linked oligosaccharides are attached to serine or threonine residues and *N*-linked oligosaccharides are attached to asparagine residues in proteins. Changes in glycosylation that correlate with cancers have been reported for both *O*- and *N*-linked oligosaccharides (glycans).[9,25] However, changes in *O*-glycans are structurally more diverse with significant modifications observed in cancers.[26,27] Different release procedures have been developed to release the two types of oligosaccharides. In this study, the reductive beta-elimination under strong basic condition was used to chemically release *O*-linked glycans. There is currently no suitable enzyme available for the same purpose. It should be noted that the beta-elimination procedure releases not only *O*-linked glycans but some *N*-glycans as well, as reported by this laboratory[18,19] and elsewhere.[28,29]

Carbohydrate and Protein Staining of SDS PAGE Separated Proteins from Conditioned Media of Ovarian Cancer Cell Lines and Human Sera. To test the feasibility and effectiveness of glycoprotein separation by gel electrophoresis and differential staining, proteins present in the conditioned media of ovarian cancer cell lines, SKOV3, ES2, OVCAR, CaOV3, and individual human sera (two ovarian cancer patients and one normal control) were separated with 6% SDS PAGE. A 6% acrylamide gel was used for this analysis because of the large glycoprotein sizes. Specific stains for total proteins (Figure 1A) and for glycoproteins (Figure 1B) show the presence of glycosylated and nonglycosylated proteins. The most abundant protein is albumin, which was observed as being heavily stained with Coomassie blue at the bottom of all samples in Figure 1A. This protein is not stained by the carbohydrate stain in Figure 1B. Most of the glycosylated proteins were 180 kDa or greater in size. At the top of each lane are glycoprotein bands (above 500 kD) found in both cell lines and human serum samples. They were identified by Western blot to contain the mucin-type cancer antigen, CA125 (Figure 1C).

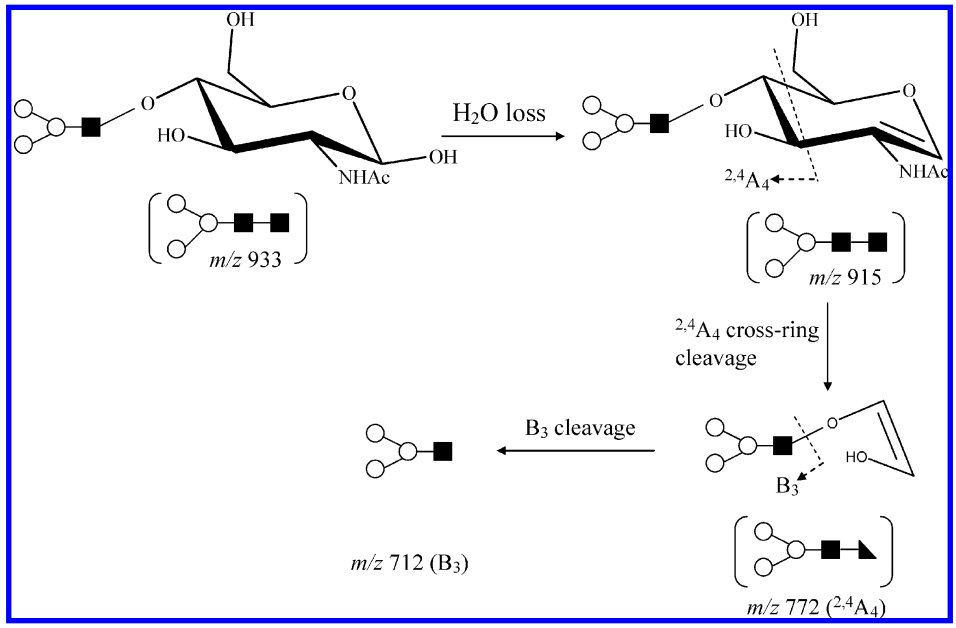
Protein bands stained for carbohydrates were removed and analyzed for oligosaccharides to determine whether sufficient quantities of the glycoproteins were present for glycan analysis. The proteins were extracted from the gel pieces by electroelution and then subjected to alkaline sodium borohydride to chemically cleave glycans by β-elimination. This approach was used for the sample from the ovarian cancer cell line, ES2. Two glycosylated protein bands of high molecular weight were selected for analysis (Figure 2A). The protein band I was chosen because it was located where CA125 was detected by immu-

Table 3. List of Positive Oligosaccharides Found in Glycoproteins of 517 kD (Apo B-100) for Normal and Ovarian Patient Sera (Figure *4*)

observed mass (*m*/*z*)	oligosaccharide composition[a]	band source (517 kD)	quasimolecular ion
347.1251	2Hex-H_2O	patient	$(M + Na)^+$
388.1605	(1Hex:1HexNAc)-H_2O	patient	$(M + Na)^+$
408.1900	(1Hex:1HexNAc)-ol	patient	$(M + Na)^+$
429.1923	2HexNAc-H_2O	patient	$(M + Na)^+$
449.2675	(1HexNAc:1HexNAc)-ol	patient	$(M + Na)^+$
509.2176	3Hex-H_2O	both	$(M + Na)^+$
591.2827	(1Hex:2HexNAc)-H_2O	patient	$(M + Na)^+$
611.3156	(1Hex:1HexNAc:1HexNAc)-ol	patient	$(M + Na)^+$
671.1638	4Hex-H_2O	both	$(M + Na)^+$
700.1723	(1Hex:2Fuc:1HexNAc)-ol	normal	$(M + Na)^+$
737.1276	(1Hex:1HexNAc:1NeuGc)-ol	normal	$(M - H + 2Na)^+$
762.1062	(2HexNAc:1NeuAc)-ol	normal	$(M - H + 2Na)^+$
771.2590	2Hex:2HexNAc	patient	$(M + Na)^+$
772.3299	(3Hex:1HexNAc:1HexNAc-^{2,4}A)[b]	patient	$(M + Na)^+$
814.3565	(1Hex:2HexNAc:1HexNAc)-ol	patient	$(M + Na)^+$
833.3155	5Hex-H_2O	patient	$(M + Na)^+$
855.2380	3HexNAc:1HexNAc-ol	normal	$(M + Na)^+$
883.2694	(1Hex:1Fuc:1HexNAc:1NeuGc)-ol or (2Hex:1HexNAc:1NeuAc)-ol	both	$(M - H + 2Na)^+$ $(M - H + 2Na)^+$
899.1884	(2Hex:1HexNAc:1NeuGc)-ol	patient	$(M - H + 2Na)^+$
915.3779	(3Hex:2HexNAc)-H_2O	patient	$(M + Na)^+$
924.2021	(1Fuc:2HexNAc:1NeuGc)-ol or (1Hex:2HexNAc:1NeuAc)-ol	normal	$(M - H + 2Na)^+$ $(M - H + 2Na)^+$
995.2418	6Hex-H_2O	patient	$(M + Na)^+$
1045.3473	(2Hex:1Fuc:1HexNAc:1NeuGc)-ol or (3Hex:1HexNAc:1NeuAc)-ol	normal	$(M - H + 2Na)^+$ $(M - H + 2Na)^+$
1157.3996	7Hex-H_2O	patient	$(M + Na)^+$
1243.4707	(4Hex:1Fuc:2HexNAc)-ol	both	$(M + Na)^+$

[a] Some *N*-linked glycans are fragments, and all *O*-linked glycans are in alditols. [b] See Scheme 2 for the cross-ring cleavage.

Scheme 2. Proposed Mechanism by Which a Pair Ions of Glycans (*m*/*z* 712/772) Which Have a Mass Difference of 60 are Related by a $^{2,4}A_4$ Cross-Ring Cleavage at the Reducing End *N*-Acetyl glucosamine (GlcNAc) of the Larger Glycan (*m*/*z* 772)[a]



[a] All ions are sodiated in mass spectrometric analysis. ○ Mannose (Man); ■ *N*-acetyl glucosamine (GlcNAc); filled right triangle $^{2,4}A_4$ residue.

noblotting (Figure 1C). Band II was selected because it represented another abundant glycoprotein present in all conditioned media of ovarian cancer cell lines. It was not present in the DMEM media with 10% FBS (lane 1 of Figure 1A and 1B).

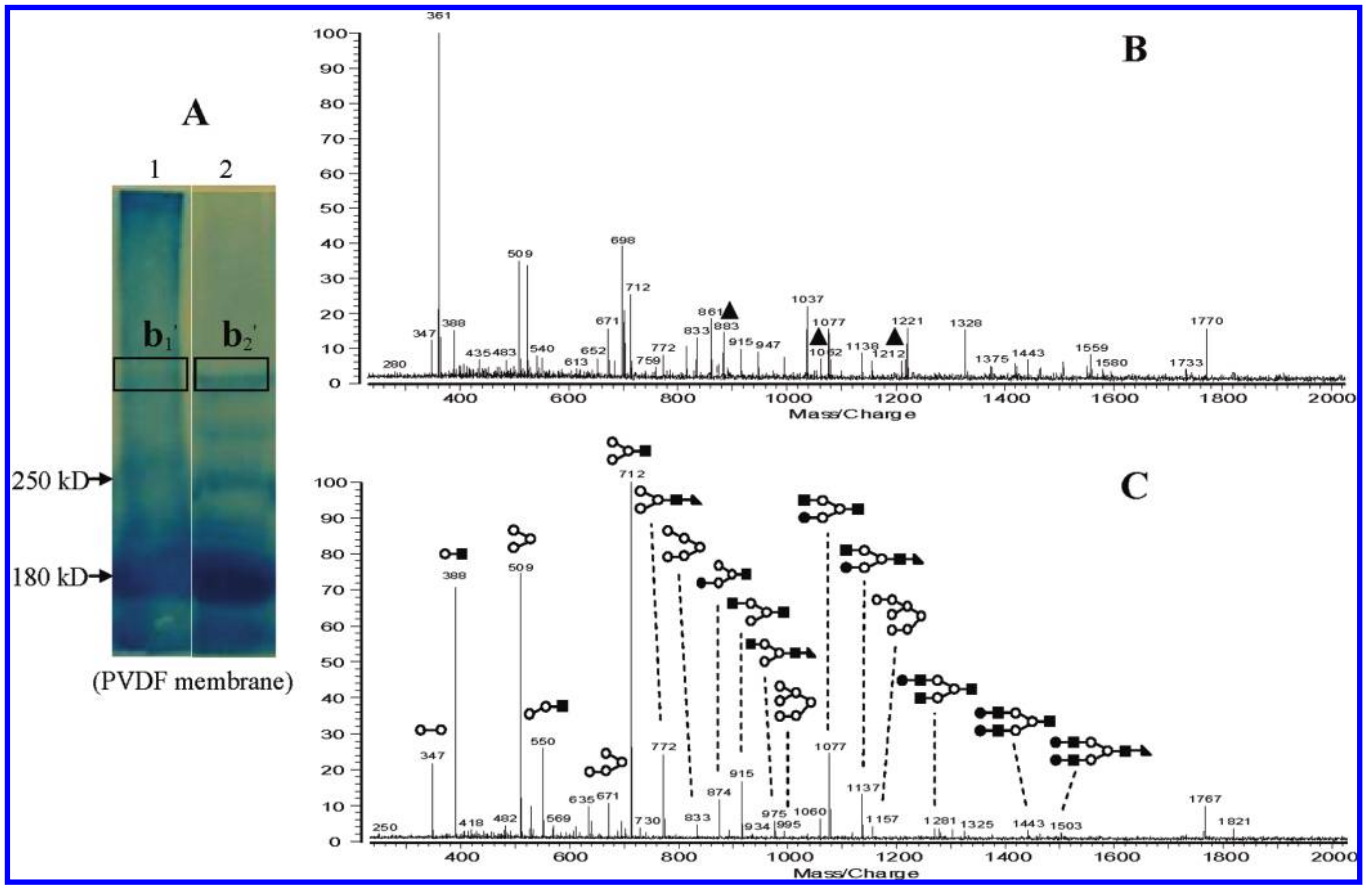


Figure 5. MALDI mass spectra of glycans released from glycoproteins of 517 kD (apolipoprotein B-100 bands, b_1' and b_2', in A) for pooled normal (B) and pooled patient sera (C). Glycoproteins were transferred on PVDF membrane from gel by using semidry electrophoretic transfer cell. The glycoprotein bands of interest on the membrane were excised into tiny pieces and then subjected to glycan release reaction. Lane 1: patient sera; lane 2: normal sera. ○ Man; ● Gal; ■ GalNAc; filled right triangle ^{2,4}A residue. ▲ specific glycans in normal sera.

The mass spectra of glycans from the bands are shown in Figure 2B and C. Based on accurate mass analysis, several glycans were observed in both bands I (Figure 2B) and II (Figure 2C). The masses, oligosaccharide compositions, and the quasimolecular ions of each mass are listed in Table 1. It was noted that all glycans observed in the positive-mode spectra were sodiated, and for acidic oligosaccharides the proton on the carboxylic acid gets replaced by Na^+ since NaCl was added as a cation dopant when MALDI sample was prepared (see Experimental Section). Therefore $[M - nH + (n + 1)Na]^+$ species were always observed under conditions used in the study.[30] Interestingly, in Figure 2B (band I), there were two sulfated glycans (m/z 453.0726 and 599.2885) both with a hexose alditol suggesting a potentially novel hexose reducing end.[27] *O*-glycans with a hexose reducing end are not common to humans but have been reported from some mammalian samples.[31,32]

It has been demonstrated and reported by An et al.[18] that the cell culture media containing 10% fetal calf bovine serum do not produce detectable oligosaccharides in experiment conditions used in the study. Therefore the presence of these glycans containing a hexose reducing end in the ovarian cancer cell line suggests unique glycosylation processes during carcinogenesis. Several of glycans were sialylated - sialylation has been reported to elevate in ovarian cancer.[33,34] Larger oligosaccharides were found from the protein in band II despite the protein's smaller mass. Many of these glycans were also sialylated and some were sulfated (Table 1).

Although the glycosylation was determined for glycoproteins in bands I and II of the cancer cell line (ES2), the protein identification was not successful for these bands. They illustrate instead that glycosylation and changes in glycosylation can be performed even at the level of individual bands. Unfortunately, the difficulty in identifying the proteins is related to the extent of protein glycosylation. Highly glycosylated proteins are often resistant to trypsin digestion - an important requirement for protein identification. However, we show below a number of proteins that are identified with changes of glycosylation readily evident.

Identification of Glycoproteins by In-Gel Tryptic Digestion and Peptide Mass Fingerprinting. Peptide mass fingerprinting was used to determine the identity of the major components in specific bands (see Table 2). While one single band in a 1-D gel is most likely made up of several proteins, it was felt that identifying the most abundant glycoprotein component would be sufficient to provide information on whether glycosylation changes could be occurring and monitored appropriately. An example of matched peptide masses for one glycoprotein was shown in Chart 1. One gel band (a in Figure 3A, approximately 250 kD) from pooled sera of ovarian cancer patients was subjected to in-gel tryptic digestion with subsequent MALDI MS analysis. Mascot database search identified proteins in the band to be a complex of fibronectin (FN) isoforms. The identification of FN was confirmed by Western blot with monoclonal antibody against FN for both

Table 4. List of Glycans Predominantly Observed in a 517 kD Band of Apo-Lipoprotein Complex of Ovarian Cancer Sera Transferred onto PVDF Membrane (Figure *5*)

observed mass (m/z) $(MNa)^+$	oligosaccharide composition	note[a]
347.0999	2Hex-H_2O	
388.1253	(1Hex:1HexNAc)-H_2O	
509.1541	3Hex-H_2O	
550.1826	(2Hex:1HexNAc)-H_2O	
671.2287	4Hex-H_2O	
712.2386	(3Hex:1HexNAc)-H_2O	
772.2699	3Hex:1HexNAc:1HexNAc-^{2,4}A	^{2,4}A at reducing end HexNAc
833.3071	5Hex-H_2O	
874.3042	(4Hex:1HexNAc)-H_2O	
915.3309	(3Hex:2HexNAc)-H_2O	
975.3740	3Hex:2HexNAc:1HexNAc-^{2,4}A	^{2,4}A at reducing end HexNAc
995.3838	6Hex-H_2O	
1077.3912	(4Hex:2HexNAc)-H_2O	
1137.4296	4Hex:2HexNAc:1HexNAc-^{2,4}A	^{2,4}A at reducing end HexNAc
1157.4421	7Hex-H_2O	
1239.5296	(5Hex:2HexNAc)-H_2O	
1280.5601	(4Hex:3HexNAc)-H_2O	
1442.5470	(5Hex:3HexNAc)-H_2O	
1502.5908	5Hex:3HexNAc:1HexNAc-^{2,4}A	^{2,4}A at reducing end HexNAc
1562.7243	5Hex:3HexNAc:1HexNAc-^{0,2}A	^{0,2}A at reducing end HexNAc

[a] Note: ^{2,4}A and ^{0,2}A are cross-ring cleavages occurring at reducing ended HexNAc. See Scheme 2 for ^{2,4}A cleavage as an example.

pooled sera of patients and normal controls (Figure 3B). Fibronectin is a high molecular weight glycoprotein found as a dimer composed of 250 kDa monomers. FN is present as two forms in human: one as plasma FN, which is soluble, and the other as cell surface FN existing in extracellular matrix, which is insoluble.[35] The reduction or loss of FN expression has been observed in many transformed cells in culture. However, overexpression of FN is associated with various human tumor cells.[36] The FN molecule is known to have six potential *N*-glycosylation sites and a number of potential *O*-glycosylation sites.[37,38]

Two other glycoprotein bands (b, c, in Figure 3A, about 510 and 370 kD, respectively) in normal pooled sera were primarily identified to be apolipoprotein B-100 precursor (Apo B-100) and its truncated form or a peptide fragment (Table 2). Apo B-100 is a glycoprotein, a major component of low density lipoprotein (LDL), and is the ligand recognized by the LDL receptor. It is initially produced in human liver and is required for the assembly of very low density lipoproteins (VLDL).[39] The mature Apo B-100 consists of 4536 amino acid residues and has an approximate molecular weight of 513 kD - consistent with the upper band (b in Figure 3A) in the gel.[40] The 370 kD protein band, identified to be a truncated Apo B-100 or a peptide fragment of Apo B-100, could be a naturally occurring fragment of mature Apo B-100 by proteolysis in human plasma. It is noted that the 370 kD glycoprotein is absent in cancer patient sera (indicated by a dotted arrow in Figure 3A), suggesting that the glycoprotein may be down-regulated in the cancer. It has been reported that one member of the apolipoprotein family, apolipoprotein A1, is down-regulated in ovarian cancer and has been suggested as a marker for early detection of the disease.[41,42]

Finally, another glycoprotein band with a molecular weight smaller than 180 kD standard (band d in Figure 3A) was identified to contain primarily immunoglobulin (Ig, 163 kD). In humans there are two subclasses of IgA—monomeric IgA1 with molecular size of ~160 kD mainly found in serum and secretory IgA2, which is IgA oligmeric (dimeric, trimeric and tetrameric), and is found in mucous secretions on most mucosal surfaces.[43,44] The molecular size and its location of the gel band d suggest that it may be monomeric IgA1, which is also confirmed by the presence of *O*-linked glycans in the glycoprotein (See below).

Changes in Glycosylation between Normal and Ovarian Cancer Sera for Selected Protein Bands. Apoliporotein B-100. The glycans were chemically released from Apo B-100 after the glycoproteins were extracted from gel bands by electroelution in both normal and ovarian cancer sera (bands b_1 and b_2 in Figure 4A). The resulting glycan mass profiles are shown in Figure 4B and 4C, respectively. To ensure the reproducibility of our glycan analysis, three similar protein bands from three separate SDS PAGE gels containing the same samples were extracted and analyzed separately. Glycan profiles from the three samples showed highly similar mass spectra (data not shown).

Glycan masses and putative compositions are summarized in Table 3. All glycans observed are sodiated. In normal sera, the Apo B-100 complex contains short-chain oligosaccharides, m/z 509.2176 (3Hex), 671.3212 (4Hex) that are less abundant and may be the fragments of larger *N*-linked glycans, and *O*-linked glycans, many of which are sialylated, specifically m/z 700.1723, 737.1276, 762.1062, 855.2380, 883.2694, 924.2021, 1045.3473 and 1243.4707.

The glycoproteins in both normal and cancer sera produced several similar glycans masses (m/z 509, 671, 883, 1243—note that for brevity, only the nominal mass will be henceforth provided, the accurate masses are provided in the tables). In ovarian cancer sera, however, the Apo B-100 band produced glycans that were distinct from those found in the normal sample. In the cancer patients, smaller *O*-linked glycans or glycan fragments (m/z 347, 388, 408, 429, 449, 591, 611, 814) were observed. These truncated glycans may be produced by the interruption of glycosylation caused by the lack of certain glycosyltransferases. We have previously reported glycans with m/z 347, 388, 509, 671, 772, 833, 915, 995, 1156 as potential markers for ovarian cancer.[18] Glycans with m/z 509 and 671 were also observed in normal sera, but they have relatively stronger signals in cancer sera suggesting that these glycans are elevated in the cancer. Indeed, specific glycans from apolipoprotein B-100 in human serum have been reported to be elevated in cancer patients compared to healthy controls.[17]

Another method was used to isolate the proteins and determine the glycosylation. In this method, proteins were transferred from the gel to PVDF membrane before releasing the glycans (see Experimental Section). The membrane was initially thought to offer the advantages of simpler and possibly more effective release of glycans. In addition, proteins could be identified by specific antibodies providing further confirmation. The mass spectra of glycans released from Apo B-100 on PVDF membrane for normal and patient sera are shown in Figure 5. From the patient sera, the Apo B-100 yielded those glycans that have been reported in this laboratory as glycan markers for ovarian cancer.[18] Apo B-100 from normal sera yielded similar peaks with weak abundances.

There are notable differences in the glycan profiles between the two approaches. With the transfer of glycoproteins to the PVDF membrane, it appears that fewer of the oligosaccharides

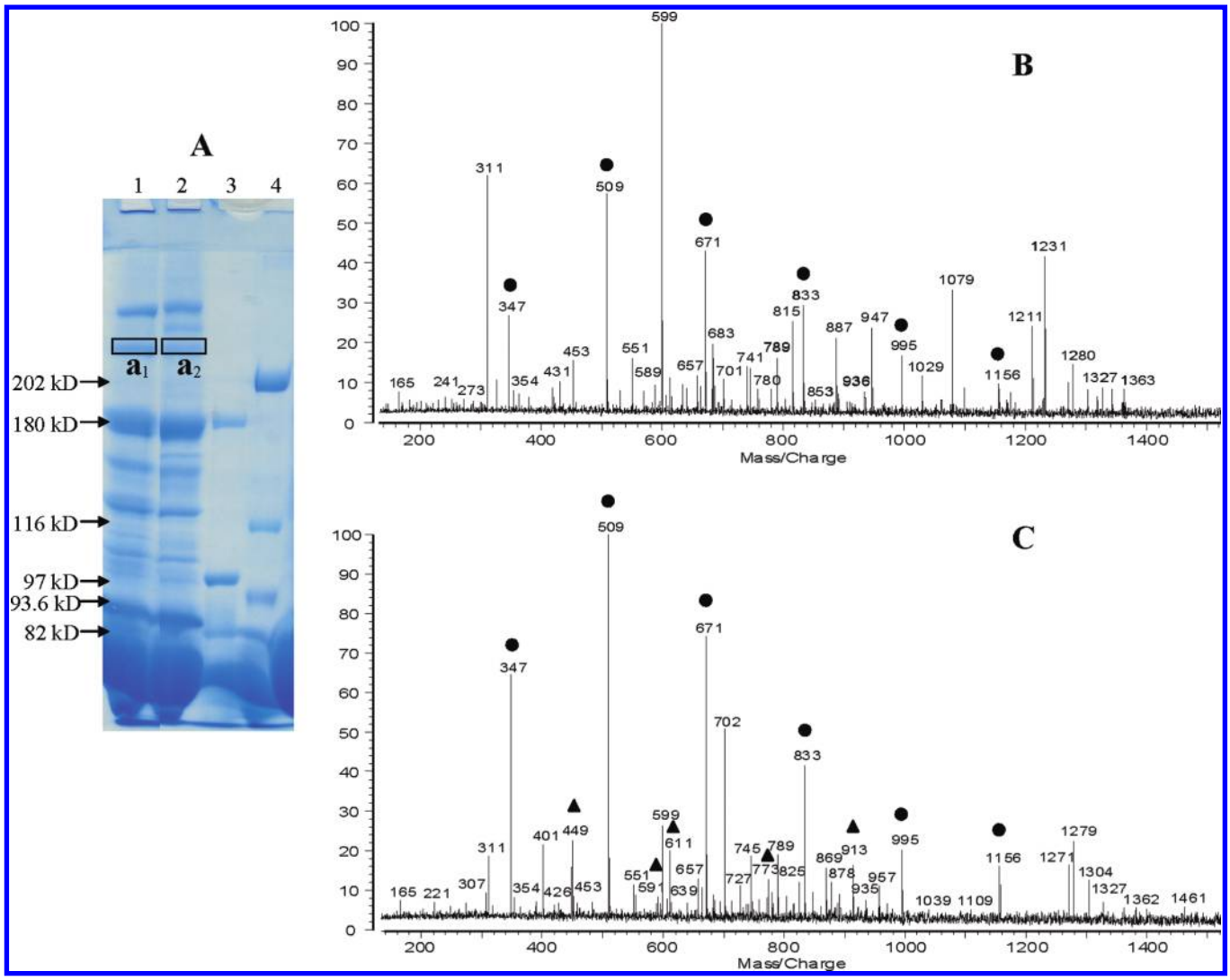


Figure 6. MALDI mass spectra of glycans released from glycoproteins of 250 kD (fibronectin gel bands, a_1 and a_2, in A) for normal (B) and patient sera (C). Glycoproteins were electroeluted out of gel bands. Lane 1: pooled patient sera; lane 2: pooled normal sera; lane 3: CandyCane glycoprotein molecular weight standards; lane 4: prestained SDS-PAGE standards. ● glycans common in normal and patient fibronectin complex; ▲ specific glycans in patient comples.

were obtained compared to those electroeluted into solution, suggesting that the transfer of glycoproteins, especially large ones, to PVDF membrane may not be highly effective as suggested by previous reports.[45,46] Putative structures of the glycans are labeled for the corresponding peaks (see Figure 5C). A summary of the glycan compositions is listed in Table 4. The glycans allow the differentiation between patients and normal controls. Some have compositions consistent with *O*-glycans but others have compositions that may be fragments of *N*-glycans. The latter have been observed in high abundances in the sera of cancer patients and may come from proteins such as Apo B-100.

We suspect that *N*-glycan fragments are present due to several unique pairs of signals in Figure 5C and Table 4, namely *m*/*z* 712 and 772, 915 and 975, 1077 and 1137, and 1443 and 1503 that have a mass difference of 60. In a previous study, we found that the reductive β-elimination of glycans from glycoproteins under basic condition releases both *O*-linked and *N*-linked glycans.[18] These signals may be due to cross-ring cleavages at the reducing end *N*-acetyl glucosamine (GlcNAc). For example, the pair of *m*/*z* 712/772 may have the relationship shown in Scheme 2. Other pairs of ions have similar relationship and are summarized in Table 4.

Fibronectin. Gel bands identified to contain fibronectin were subjected to electroelution to extract the glycoprotein (Figure 6A) followed by glycan release and MS analysis. Representative mass spectra for normal controls and ovarian cancer patients are shown in Figure 6B and C, respectively. Interestingly, both bands yield a series of hexose oligosaccharides (*m*/*z* 347, 509, 671, 833, 995, 1156) with higher intensities in patients. There are unique glycans from patients such as *m*/*z* 449 [1HexNAc:1HexNAc-ol+1Na]$^+$, 591 [1Hex:2HexNAc+1Na]$^+$, 611 [1Hex:1HexNAc:1HexNAc-ol+1Na]$^+$, and 773 [2Hex:1HexNAc:1HexNAc-ol+1Na]$^+$. The compositions correspond to *O*-linked oligosaccharides that are not present in the normal controls. Ueda et al. have reported that some glycosylation levels in human serum fibronectin are increased in cancer.[17] Fibronectin in gel bands was also transferred to a PVDF membrane, and fewer glycans were observed (data not shown).

Immunoglobulin (Ig). The glycoprotein bands at 163 kD (d in Figure 3A) were preliminarily identified as serum IgA1 based on database searching and its location in the gel. IgA1 in

ovarian cancer sera has glycans distinct from normal control sera (mass spectra not shown here). The glycoprotein in cancer sera has unique glycans with m/z 388, 449, 550, 611, 712, 772, 875. It also has glycans in common with the normal sera including m/z 347, 509, 671, 833, 995, 1157, 1304 and m/z 741, 887, 1029, 1211. The glycans in the latter group have *O*-glycan compositions. The compositions of these *O*-glycans are proposed as follows: m/z 741 with [2Fuc:1HexNAc:1GalNAc-ol+1Na]$^+$, m/z 887 [3Fuc:1HexNAc:1GalNAc-ol+1Na]$^+$, m/z 1029 [2Hex:1Fuc:1GalNAc-ol:1NeuAc-1H+2Na]$^+$ and m/z 1211 [2Hex:3Fuc:1HexNAc:1GalNAc-ol+1Na]$^+$.

IgA would not be produced by the ovarian cancer cells, but IgA is the major immunoglobulin found in mucous secretions that include tears, saliva, colostrum, intestinal juice, vaginal fluid and secretions from the prostate and respiratory epithelium. Evidence that IgG, IgA and IgM are produced in response to ovarian cancer antigens has been reported.[47] The glycan compositions are consistent in distinct core structures with previously identified Ig glycans.[48,49] The *O*-glycans come from two partially occupied sites, Thr^{225} and Thr^{236}, and three conserved and fully occupied sites, Thr^{228}, Ser^{230} and Ser^{232}.[48–50] Some of the glycans (m/z 388, 550, 712, 772) unique to patients may be fragments (refer to Figure 5C) of larger *N*-linked sugars, while others have compositions consistent with *O*-glycans m/z 449 [1HexNAc:1GalNAc-ol+1Na]$^+$, 611 [1Hex:1HexNAc:1GalNAc-ol+1Na]$^+$, and 875 [2Hex:1HexNAc:1GalNAc-ol:1Sulfate-1H+2Na]$^+$. They are also consistent with the glycans identified as markers in a previous report from this laboratory.[18]

Conclusion

Several glycoproteins were identified that could be derived from the cancer. They were found to contain aberrant glycosylations. Even in proteins that were not produced in the cancer such as IgA1, aberrant glycosylation was found perhaps as a response to the cancer. The results support the conjecture that in cancer patients, several proteins can be simultaneously misglycosylated. This condition provides a unique opportunity and also points to the potential of glycans as cancer markers.

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